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Evolution

A molecular phylogeny of the hominoid primates as indicated by two-dimensional protein electrophoresis

(genetic distance)

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ABSTRACT **A molecular phylogeny for the hominoid primates was constructed by using genetic distances from a survey of 383 radiolabeled fibroblast polypeptides resolved by two-dimensional electrophoresis (2DE). An internally consistent matrix of Nei genetic distances was generated on the basis of variants in electrophoretic position. The derived phylogenetic tree indicated a branching sequence, from oldest to most recent, of cercopithecoids (*Macaca fascicularis*), gibbon–siamang, orangutan, gorilla, and human–chimpanzee. A cladistic analysis of 240 electrophoretic characters that varied between ape species produced an identical tree. Genetic distance measures obtained by 2DE are largely consistent with those generated by other molecular procedures. In addition, the 2DE data set appears to resolve the human–chimpanzee–gorilla trichotomy in favor of a more recent association of chimpanzees and humans.**

The resolution of human evolutionary history has long fascinated paleontologists and naturalists and more recently has provided an arena for the development of the fields of molecular anthropology (1, 2) and molecular evolution (3–5). Sarich and Wilson (6) derived a molecular topology based upon immunological distance between serum albumins. Their transformation of albumin immunological distances was based upon the molecular clock hypothesis (7). The theory states that the extent of DNA or protein sequence divergence reflects relative evolutionary distance and elapsed time since sharing a common ancestor. By utilizing "outgroup" species outside a phylad, the relative constancy of the clock's rate can be tested. The generally (but not universally) found result is that molecules diverge stochastically and that, at least within the linear range of any particular molecular method, the molecular clock can be a fairly accurate timekeeper (3–8).

The molecular evolution of the hominoid primates has been studied by DNA hybridization, DNA sequencing, albumin/transferrin immunological distance, isozyme genetic distance, and mitochondrial DNA restriction maps. In spite of certain contradictions, it is striking how concordant the derived phylogenies are with each other and with the limited fossil evidence for this group (6, 8–13). In general, the great and lesser apes are thought to have split from the Old World monkeys, Cercopithecidae, approximately 30–40 million years (Myr) before the present. The next split, which led to the lesser apes, Hylobates, was followed by the divergence of the ancestors of the Asian great ape, the orangutan. The Hominidae split from the African apes as recently as 4.5 Myr ago (5, 6). The trichotomy of chimpanzee–human–gorilla was not resolved by albumin, isozyme, or early DNA hybridization studies. Sibley and Ahlquist (13) have argued that their DNA hybridization results favor a chimpanzee–human association after a gorilla divergence, a conclusion that was also supported by karyological analysis (14). However, comparison of restriction maps of mitochondrial DNA suggested a more recent association of gorilla and chimpanzee after their split from the human line (12, 15). Finally, phenotypic arguments have been raised that dismiss the human–African ape association and conclude that the closest living relative of humans is the orangutan (16).

In this report we provide another estimate of genetic distance between humans and the Pongidae primates. Using two-dimensional electrophoresis (2DE), we followed 383 fibroblast proteins labeled with [^{35}S]methionine for each species. Several algorithms for phylogenetic tree construction were employed (17–23). The results produce an apparent resolution of the human–gorilla–chimpanzee trichotomy and tend to affirm conclusions based on other molecular distance data for the hominoid primates.

MATERIALS AND METHODS

Primary fibroblast lines were established from skin biopsy samples of the crab-eating macaque (*Macaca fascicularis*), gorilla (*Gorilla gorilla*), two chimpanzees (*Pan troglodytes*), siamang (*Symphalangus syndactylus*), crested gibbon (*Hylobates concolor*), and two Sumatran orangutans (*Pongo pygmaeus abelii*). Human cell lines (*Homo sapiens*, GM3234, GM3433, GM3349, GM726, and GM5294) were from the Institute for Medical Research (Camden, NJ). Dividing fibroblasts were labeled with [^{35}S]methionine for 3 hr as described (24, 25). Proteins were extracted and separated on two-dimensional gels. Procedures for analysis of derived autoradiograms, including quantitative densitometry, are presented elsewhere (24–26). Nei genetic distance (27) was computed using a modification for distance estimates based on low numbers of individuals (28). Because 2DE permits accurate determination of single-charge versus double-charge shifts, the raw data were weighted accordingly. This convention, proposed by Nei (27) and King (29), has been virtually ignored in previous estimates of genetic distance using 2DE (30, 31). The use of weightings is based on the premise that double-charge shifts are due to double mutations about 99% of the time (27). This idea was affirmed by the present results. In 35 of 37 cases (95%) in which we observed electrophoretic forms separated by more than one charge, another primate showed the intermediately charged form. The weightings used were as follows: single-charge shifts, 1; double-charge shifts, 2; heterozygous, 0.5; missing or extra polypeptide, 0.5; and shifts in molecular weight, 1.0. Heterozygous loci were detected, and in the case of the human sample, overall heterozygosity was close to levels determined by this laboratory (24, 25) and by others (32, 33).

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Abbreviation: 2DE, two-dimensional electrophoresis.

RESULTS

Fibroblasts from five humans, two chimpanzees, two orangutans, and single individuals of gorilla, crested gibbon, siamang, and crab-eating macaque were labeled with [^{35}S]methionine and subjected to 2DE. The positions of 383 human proteins are presented in Fig. 1. Protein charge variants were detected in autoradiograms by observing the absence of a polypeptide in one species accompanied by a new polypeptide in the approximate position expected for charge substitutions at that molecular weight. The distribution of protein variation among the seven primates' proteins was not random. As had been observed for isozyme variation (34), there is a group of loci (60%) that were invariant across all seven species. This background of phylogenetically uninformative proteins provided landmarks for identifying electrophoretic shifts of putatively homologous proteins. The majority of differences were charge shifts. Most proteins that showed charge variation occurred in one of two isoelectric mobilities. Of the 383 polypeptides, only 35 had three or more isoelectric forms, and only seven exhibited shifts in molecular weight.

The only variation observed at 40 loci was that a polypeptide observed in one species was missing in another. This situation would be explained by (*i*) a charge shift that caused a protein to comigrate with another protein or away from the region of observation; (*ii*) shift in molecular weight making assignment difficult; (*iii*) dramatic alteration of quantitative expression of the locus. The occurrence of notable quantitative variation in the expression of several polypeptides is consistent with this latter explanation. We approached the contribution of this category of variation by performing parallel analyses (see below), with and without the loci that apparently are missing polypeptides. The influence of these variants on the final computations was slight, insofar as the correlation coefficient of distances computed for loci with missing polypeptides only versus all 383 loci was 0.92. Further, the derived phylogenetic topologies were nearly identical.

Genetic distances computed with all 383 loci and with 330 loci in which none of the species were missing the polypeptide are presented in Table 1. Prior to actual tree construction, the data were judged as adequate in two ways. First, the principle of the triangle inequality (17) states that a distance matrix is "metric" when the sum of the two distances between any one species (*A*) and any two other species (*AC* and *AB*) exceeds the distance between the two other species (*BC*). Of the 35 three-way comparisons in the $N = 383$ data set, none violated the triangle inequality. As stated by Farris (36), a distance may be metric but the rate of the evolutionary clock can still vary between lineages. The constancy of the 2DE evolutionary clock was evaluated by demonstrating that related species were equidistant from an out-group, the relative rate test (5, 6). The outgroup, the macaque, had a mean distance of 1.12 from the other species (range 1.01–1.4). Closer examination of the data showed that the siamang was inexplicably more divergent from all species than was the crested gibbon. If the siamang is not considered, the mean

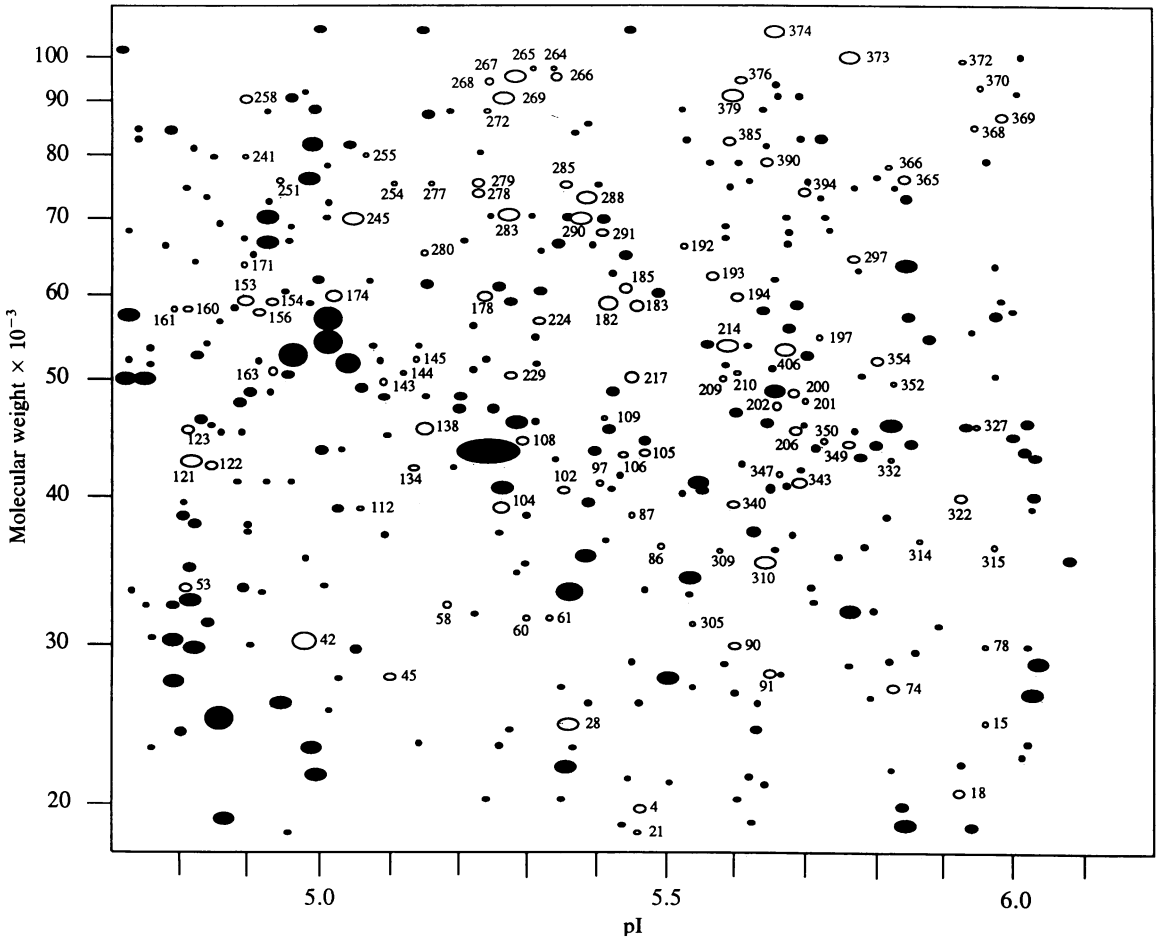

FIG. 1. Relative positions and concentrations of the 383 human fibroblast proteins analyzed. Open symbols, proteins that varied within or between primates examined; the variable proteins listed in Table 2 are numbered. Solid symbols, proteins invariant among all primates examined. Symbol areas are proportionate to density (concentration) of individual proteins. Photographs of human fibroblast protein gels are presented elsewhere (25).

Table 1. Genetic distance (D') derived from comparison of 383 fibroblast proteins

	Human	Chimpanzee	Gorilla	Orangutan	Siamang	Crested gibbon	Crab-eating macaque
Human	—	0.070	0.097	0.118	0.173	0.158	0.330
Chimpanzee	0.062	—	0.107	0.103	0.166	0.147	0.303
Gorilla	0.085	0.094	—	0.115	0.184	0.143	0.309
Orangutan	0.098	0.088	0.097	—	0.175	0.153	0.327
Siamang	0.158	0.157	0.165	0.158	—	0.100	0.420
Crested gibbon	0.138	0.137	0.121	0.139	0.094	—	0.333
Crab-eating macaque	0.267	0.245	0.248	0.252	0.360	0.265	—

Nei genetic distances (D') were derived by correcting D values for back mutation and small sample size (see text). The fraction of amino acid substitution detectable by electrophoresis has been estimated to be 0.3 (35). Heterozygosity (h) for each species was assumed to be equivalent to human values (h = 0.02; ref. 24), because examination of a low number of individuals may be likely to underestimate this value (28). Numbers above and to the right of the diagonal represent all loci; N = 383. Numbers below and to the left of the diagonal represent loci excluding those that had "missing or extra" polypeptides in one or more species; N = 330.

distance between macaque and apes is 1.07 with a narrower range of 1.01–1.11. Because the great apes diverged at a time after the gibbon–siamang divergence, the gibbon can be considered as an additional out-group. The crested gibbon has a mean distance of 0.50 from the great ape species (range 0.49–0.52). With the stated reservation of the apparently accelerated siamang rate of divergence, the data set conforms well to the expectations of a steady and stochastic clock as evaluated by the relative rate test.

Phylogenetic trees were derived from genetic distances by using five algorithms: the distance-Wagner procedure of Farris (17); the UPGMA algorithm of Sneath and Sokal (19); the "neighborliness" method of Fitch (20); the MATTOP program of Dayhoff (21); and the parsimony method of Fitch and Margoliash (22). The latter method clusters the closest taxa and the next closest taxon is found by minimizing the sum of the squares of deviations between the experimentally determined distances and the patristic distances computed for the phylogenetic tree. The Fitch–Margoliash trees for the two distance matrices (N = 383 and N = 330) in Table 1 are presented in Fig. 2. The time scale is calibrated after Andrews (37), who places the orangutan divergence at approximately 13 Myr ago. All of the algorithms produce similar trees, which differ largely in leg lengths. However, most algorithms could not exclude a tree that places the orangutan and gorilla divergences as simultaneous.

The derived topology is not at variance with conventional molecular and paleontological conclusions for this group. The earliest event was the split of the Old World monkeys 35+ Myr ago. The orangutan diverged next, but gorilla split

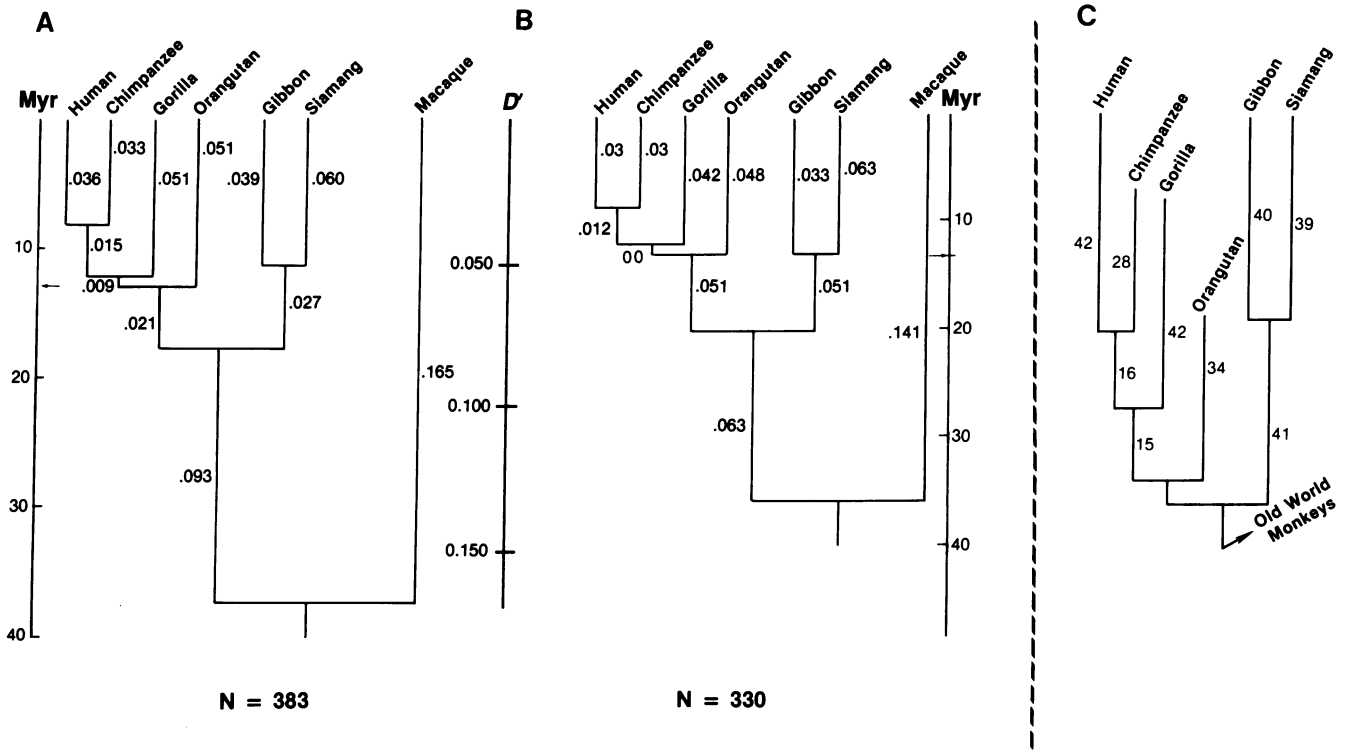

FIG. 2. (*A* and *B*) Phylogenetic trees were derived by using the Fitch–Margoliash algorithm (22) and the D' genetic distances presented in Table 2 and computed as discussed in the text. Topologies were drawn to scale by using KITSCH of the PHYLIP program generously provided by J. Felsenstein (University of Washington, Seattle). This program computes a rooted topology based upon the assumption of a constant rate of molecular substitution in all lineages. The leg lengths were generated in the absence of the above assumptions (22). This tree is rooted at the midpoint between the two most distal species in the "network." (*C*) Phylogenetic tree derived from maximum parsimony analysis using 240 protein character states from 153 loci that showed variation within the apes. For this analysis, all possible topologies were evaluated by the PAUP program made available by D. L. Swofford (Illinois Natural History Survey, Champaign, IL). Branch and internodal distances are proportional to the number of character state changes required. Total length was 297 and the consistency index was 0.808.

not much later and possibly at the same time. The data clearly affirm the association of chimpanzee and human. By the neighborliness algorithm of Fitch (20), the chimpanzee and human consistently tie for best neighbors with gibbon and siamang in four-way comparisons. This was not the case with DNA hybridization or isozyme genetic distances of the same primates (9).

Finally, the 240 protein phenotypes at the 153 loci that varied among the ape species were treated as discrete characters in a cladistic analysis (Table 2) using the PAUP program of Swofford (23). All possible phylogenetic trees were evaluated and characters were unordered. The most parsimonious topology (Fig. 2*C*) is very similar to the 2DE genetic distance trees, except that the divergence of gorilla is clearly resolved as having occurred after that of the orangutan. This tree has a "length" of 297 changes and a consistency index of 0.808. The consistency index is the fraction of total changes that are not reversals (homoplasy); ideally, it should be 1.0. The alternative tree in which gorilla and chimpanzee are closest relatives had a length of 307 and a consistency index of 0.782. The tree that groups human and gorilla most closely had a length of 299 and a consistency index of 0.803.

DISCUSSION

The application of 2DE provides a genetically independent data set for the phylogeny of hominoid primates. Previous isozyme studies used an average of 22 loci, range 14–38 (38). Since different proteins evolve at different rates, the tenfold increase in the number of typed electrophoretic loci by 2DE would advantageously tend to normalize the variance between discrete loci (39, 40). This benefit may be offset, however, by limitations in establishing genetic homology between proteins resolved by 2DE. Isozyme homologues are identified by clear and rigorous enzymatic criteria (41), whereas individual proteins on 2DE gels are not. Nevertheless, the frequency of erroneous presumed homology should be much less than the 39% of the proteins that vary between species and probably is equivalent to or less than the 14% (53 of 383 proteins) that exhibit unexplained "missing spots." It is encouraging that the analysis of only those proteins that did not display "missing spots" gave the same phylogeny as did the total protein analysis (see Fig. 2).

Table 2. Relative electrophoretic mobility phenotypes of proteins that varied within the sampled primate species

Pr. no.	HSA	PTR	GGO	PPY	HCO	SSY	MFA
004	0	0	8	6	M	M	6
015	0	0	0	0	2	2	0
018	0	0	8	0	0	0	0
021	0	0	2	0	0	0	-
028	0	0	0	2	0	0	0
042	0	0	0	0	9	8	2
045	0	8	8	6	8	8	8
053	0	H	H	0	H	H	0
058	9	0	0	0	0	0	0
060	0	0	M	8	8	8	-
061	0	0	0	0	0	9	0
074	9	9	0	9	0	0	-
078	0	0	8	0	0	0	8
086	0	8	0	0	2	M	2
087	0	M	0	0	2	2	0
090	0	0	0	0	0	0	2
091	0	M	M	M	8	8	-
097	0	0	0	0	8	8	0
102	0	0	8	0	0	0	0
104	9	8	9	9	0	8	8
105	1	-	0	8	M	M	M
106	0	6	0	8	H	H	8
108	0	0	0	0	0	0	8
109	0	0	0	0	2	2	0
112	0	0	M	6	6	6	M
121	0	0	0	8	0	0	0
122	0	0	0	0	0	0	8
123	0	L	0	0	0	0	0
134	0	0	0	0	0	9	0
138	1	6	6	6	8	8	8
143	0	0	0	0	8	0	0
144	0	0	8	8	2	2	2
145	9	0	0	-	0	8	2
153	0	9	0	0	0	0	2
154	0	9	0	0	0	0	2
156	0	L	0	0	0	0	0
160	0	0	8	0	8	M	0
161	0	2	0	0	0	0	0
163	0	0	0	0	0	8	8
171	0	8	8	0	8	8	0
174	0	8	2	0	3	9	4
178	0	0	0	0	0	0	2
182	9	0	0	0	0	0	0
183	0	0	0	0	8	8	0
185	0	0	0	0	0	0	2
192	0	0	8	8	6	8	8
193	0	0	8	0	0	0	2
194	0	0	0	0	2	0	0
197	0	0	0	9	0	8	0
200	0	0	0	0	8	8	0
201	0	0	0	0	8	8	0
202	0	0	0	0	2	0	0
206	9	0	8	0	0	0	0
209	9	0	8	8	8	0	0
210	0	0	0	0	2	0	0
214	0	0	0	0	0	8	8
217	0	0	0	0	0	9	0
224	0	1	0	0	0	0	0
229	0	L	M	L	L	M	L
241	0	0	2	0	0	2	8
245	9	0	0	0	0	0	0
251	0	L	0	0	8	8	M
254	0	0	0	0	2	0	0
255	9	0	0	0	0	0	0
258	0	2	0	0	0	0	M
264	0	0	0	0	2	0	8
265	0	0	0	0	2	0	0
266	0	0	0	0	8	8	2
267	0	9	8	0	0	8	8
268	0	0	0	0	0	8	0
269	1	0	0	0	0	0	0
272	0	8	8	8	8	8	8
277	0	0	0	0	2	0	0
278	0	2	2	2	2	2	0
279	0	2	2	2	2	2	0
280	0	0	8	0	0	M	0
283	0	0	0	0	0	8	0
285	0	0	0	M	0	0	9
288	0	0	0	0	0	2	0
290	0	0	2	2	8	2	2
291	0	0	2	M	8	2	2
297	1	0	0	0	0	2	0
305	0	0	0	2	0	0	0
309	0	0	0	M	8	M	2
310	0	0	0	M	2	0	0
314	0	0	0	2	8	0	0
315	0	2	0	2	M	0	-
322	9	0	2	0	0	0	0
327	0	0	0	2	0	0	0
332	0	M	0	2	2	2	0
340	0	0	0	8	2	2	0
343	9	1	0	1	0	2	0
347	0	0	0	8	0	0	0
349	0	0	0	H	8	8	0
350	0	0	9	0	0	8	0
352	0	0	2	0	0	0	0
354	0	0	0	0	2	2	0
365	0	0	0	2	2	2	2
366	0	0	0	0	8	8	0
368	0	8	8	-	8	8	8
369	0	0	0	2	0	0	0
370	0	0	0	0	8	0	0
372	9	0	2	2	2	2	M
373	0	0	0	0	8	8	2
374	9	2	0	0	9	9	0
376	1	0	8	1	0	0	8
379	9	0	8	0	9	0	2
385	0	0	2	8	0	0	2
390	0	M	0	0	8	0	0
394	0	8	0	8	8	8	8
406	9	0	0	0	2	2	4
431	M	0	M	8	0	0	M
433	M	M	M	2	0	8	M

Pr. no., protein number; HSA, *Homo sapiens*; PTR, *Pan troglodytes*; GGO, *Gorilla gorilla*; PPY, *Pongo pygmaeus abelii*; HCO, *Hylobates concolor*; SSY, *Symphalangus syndactylus*; MFA, *Macaca fascicularis*. Electrophoretic phenotypes: 0, most common phenotype; 2, one step more acidic (than 0); 4, two steps more acidic; 8, one step more basic; 6, two steps more basic; 1, 0/2 heterozygote; 3, 2/4 heterozygote; 9, 0/8 heterozygote; 7, 8/6 heterozygote; L, lower in molecular weight; H, higher in molecular weight; M, missing; -, unscorable for technical reasons.

2DE has been used extensively in human and population genetics (24–26, 32, 35, 42), but it has seen limited application in evolutionary studies (30, 31). The approach and analysis presented here provide several improvements over these earlier studies. First, more proteins were examined (383 compared to 100 in a *Drosophila* and 189 in a rodent study) (30, 31). Second, the expected incidence of back mutations was included in the computation (27–29). Third, familiarity with 2DE patterns of human fibroblasts permitted detection of multistep electrophoretic shifts. Further, the previous identification of some 17 distinct human polymorphic fibroblast loci provided a genetic basis for the presumption that the proteins scored are single gene products. Fourth, the results were evaluated as genetic distance plus by cladistic analysis of individual characters. Because of innate difficulties associated with each molecular distance method and with the molecular clock hypothesis itself (4, 5, 29, 43, 44), a derivation of phylogenies by a consensus of several approaches offers promise, and the addition of a reliable new procedure can only help in resolving ambiguities.

For calibration of the genetic distances, we used a dating for the orangutan divergence of 13 Myr ago, based on recent interpretation of the fossil record (37). Averaging the distances of orangutan from human, gorilla, and chimpanzee, we obtain an average rate of amino acid substitution resulting in charge alteration of 0.86% per Myr. This rate is approximately one-third of evolutionary rates for isozymes, which range from 2.1% to 2.6% per Myr (8, 9). Apparently slower rates of 2DE protein divergence were also seen in mice (30) and *Drosophila* (31). Heterozygosity levels by 2DE are also about one-third of heterozygosity levels by isozyme analysis (24, 25), suggesting that the sampled 2DE loci as a group evolved more slowly than the normally typed enzyme loci. The derived phylogeny (Fig. 2) indicates that the Old World monkeys diverged approximately 37 Myr ago. The gibbons split 20–25 Myr ago. The divergence of the orangutan is placed just prior to that of the gorilla. The chimpanzee split from humans about 8 Myr ago.

The phylogenetic trichotomy among chimpanzee–gorilla–human has been vigorously debated (6, 8–14, 43, 45). Several molecular data sets were equivocal, including albumin immunological distance (6), DNA hybridization (11), isozyme genetic distance (8, 9), and mitochondrial DNA restriction maps (10). Using DNA hybridization, Sibley and Ahlquist (13) claim to have unequivocally resolved the trichotomy in favor of a recent chimpanzee–human association. High-resolution G-banded chromosome analysis (14) also supports a human–chimpanzee association. Templeton (15) reanalyzed the mitochondrial data of Ferris *et al.* (10) and concluded that chimpanzee and gorilla evolved together after splitting from the human lineage. The 2DE results presented here support the close association of the three species but consistently indicate that the chimpanzee–human lineage diverged subsequent to the split leading to the gorilla.

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